REMARKS

This amendment is submitted in an earnest effort to bring this case to issue without delay.

Applicant wishes to reiterate his claim to the benefit of his Hungarian priority date of 1 February 1999 pursuant to the International Convention. A certified copy of Hungarian Patent Application P9900213 filed 1 February 1999 has been made of record as part of Applicant's PCT HU 00/00009 filed 28 December 2000 of which the instant application is the U.S. National Phase. The Examiner is requested to acknowledge Applicant's perfected right of priority.

Applicant has canceled claims 1 through 8, 14, 17 and 18, and amended claims 9 through 13, 15 and 16. Applicant has also added new claims 19 through 25. Antecedent basis for the amendments to claims 9 through 13, 15 and 16 may be found in the specification on pages 3 through 6. Antecedent basis for new claims 19, 20, 23, 24 and 25 may be found on pages 3 through 6 and in Examples a, b and c as well where patients who are otherwise healthy are undergoing contraception treatment. Thus claims 9 through 13, 15, 16 and 19 through 25 are now in this application and are presented for examination.

The Examiner has rejected claims 10, 13 and 16 through 18 last presented under 35 USC 112, second paragraph, as indefinite. It is believed that all claims now presented are entirely definite

and that no such rejection should be maintained against any claim now presented. Claims 17 and 18 have been canceled without replacement. Claims 10, 13 and 16 have been amended to delete "metabolic precursor, analog or derivative thereof", the expression which the Examiner considers to be indefinite.

The Examiner has rejected claims 9, 13 through 15, 17 and 18 under 35 USC 112, first paragraph, as beyond the scope of the enabling disclosure. The Examiner argues that the specification is not clear as to what is meant by "plasma homocysteine content reducing agent." Furthermore the Examiner argues that the specification does not enable one "skilled in the art" how to determine what compounds or agents would in fact possess the ability to reduce plasma homocysteine content without the need to conduct undue experimentation. The Examiner believes that Applicant should limit his claims to the specific plasma homocysteine content reducing agents disclosed in the specification and in present claim 10.

Applicant did not coin the terms "elevated plasma homocysteine concentration" or "plasma homocysteine content reducing agent." These terms were well known to those skilled in the art at the time that the present application was constructively reduced to practice by the filing of the Hungarian priority application on 1 February 1999. See Welch, G.N., Loscalzo, J., :Homocysteine and Atherothrombosis, New Eng. J. Of Med. 1998, 338, 1042 to 1050 cited

on page 3 of the present application and made of record on PTO-1449 as reference (AO) and considered by the Examiner on 24 June 2004. The term "elevated plasma homocysteine concentration" is defined as homocysteine plasma concentration of at least 15μmol/liter, preferably at least 30μmol/liter and more preferably at least 100μmol/liter. See page 1042, right-hand column of the reference. On page 1046 of the reference a number of specific compounds effective in reducing homocysteine plasma concentrations are indicated.

Applicant is enclosing several publications to establish that one "skilled in the art" will be able to determine if any particular compound is an effective agent to reduce plasma homocysteine levels in patients with hyperhomocysteinemia without the need to carry out undue experimentation. In the enclosed Abbott Diagnostics Division, European Evaluations IMX, Homocysteine which was published in 1999, a collection of publications is included to show the use of the fully automated ABBOTT IMX Homocysteine Assay for the determination of plasma homocysteine level. The assay is fluorescence polarization immunoassay that is done with a kit. The assay is quick, inexpensive, and widely used both for animal tests and for humans at the patient's bedside.

The ABBOTT reference confirms that the determination of the plasma homocysteine level of a patient who may have hyperhomocysteinemia is a routine analysis that may be practiced by

those skilled in the art without the need to carry out undue experimentation. It is also routine for one "skilled in the art" to use the ABBOTT IMX Homocysteine Assay to determine whether a compound possesses homocysteine plasma level reducing activity. In such a test the plasma homocysteine level is determined both before and after administration of the test compound and the homocysteine plasma levels compared before and after said administration.

The ABBOTT IMX Homocysteine Assay is not the only way to determine the plasma homocysteine level of a patient who may have hyperhomocysteinemia and to determine whether a compound possesses homocysteine plasma level reducing activity. HPLC has been used for many years to achieve these ends. In the ABBOTT reference the ABBOTT assay is compared with assays based upon HPLC which has routinely been used for this purpose.

Applicant is enclosing two references, namely, Clin Chem. 40/6, 873 to 881 (1994) and J. Of Chromatography, 422 (1987), pp 43 to 52 to further establish that HPLC has been used routinely for many years to determine the homocysteine plasma level in patients who may have hyperhomocysteinemia. Thus there is no reason why one "skilled in the art" having the present application before him would not be able to routinely determine if any particular compound has the ability to reduce homocysteine levels in plasma without the need to conduct undue experimentation.

Therefore Applicant should not be required to limit his claims to cover only the specific compounds disclosed in his application that have been established to reduce homocysteine plasma levels in patients. Not only has Applicant shown that the state of the art includes well-known assays to determine the plasma level of homocysteine, both before and after the administration of a compound that may be useful to this end, but furthermore there are other specific compounds that are known in the art to have the ability to lower the plasma level of homocysteine. Penicillamine is a known reducer of plasma homocysteine levels. See Kang, SS, Wong, PW, Glickman, PB et al, Protein-Bound Homocysteine in Patients with Rheumatoid Arthritis undergoing D-Penicillamine Treatment, J. Clin. Pharmacol. 1986, 26:712 to 715.

There are also nucleoside analogs that are known to reduce plasma homocysteine levels. See Kredich, NM, Hershfield, MS, Falletta, JM, Kinney, TR, Mitchell, B and Koller, C, Effects of 2'-deoxycoformycin on homocysteine metabolism in acute lymphoblastic leukemia, Clin, Res. 1981, 29:541A.

Thus the specification as filed should enable one "skilled in the art" to determine if any particular compound has the ability to reduce homocysteine plasma levels without the need to conduct undue experimentation. Thus all claims now presented are based upon a specification that adequately describes the present invention and adequately enables one skilled in the art to

practice the invention. Thus no rejection of any claim now presented should be maintained under 35 USC 112, first paragraph.

The Examiner has rejected claims 9, 10 and 12 through 18 as anticipated under 35 USC 102(b) in view of the SPELLACY et al reference which discloses the administration of Vitamin B_6 as a supplement together with progesterone to women taking the progesterone orally as a contraceptive. The Examiner has also rejected claims 9 through 11 and 13 through 18 as anticipated under 35 USC 102(b) in view of the BUTTERWORTH et al reference which discloses the administration of folic acid as a supplement together with progesterone to women taking the progesterone orally as a contraceptive.

Applicant has amended independent claims 9 and 15 as well as dependent claims 10 through 13 and 16 and it is believed that none of these claims is anticipated by either by the SPELLACY et al or BUTTERWORTH et al reference. Furthermore Applicant has submitted new claims 19 through 25 and it is believed that none of these claims is anticipated by SPELLACY et al or BUTTERWORTH et al. Claims 14, 17 and 18 have been canceled without replacement.

Independent claim 9 and dependent claims 10 through 13 and new dependent claim 21 as well as independent claim 15 and dependent claim 16 and new dependent claim 22 have been drafted so that the patient receiving the combination of progesterone and a supplement is not a woman receiving progesterone as an oral contra-

ceptive. Claim 9 defines the patient as a patient undergoing treatment with a gestagen hormone composition for hormone replacement therapy, for inflammation, for an in vitro fertilization program, for dermatological therapy or for cosmetological treatment. Such a patient does not include a woman orally taking a progesterone for contraception. Claims 10 through 13 and 21, dependent upon claim 9, are also so limited. Claim 15 has been amended so that the patient whose risk of thromboembolism induced by a gestagen hormone is reduced thanks to also taking a therapeutically effective amount of a plasma homocysteine reducing agent, does not include a woman orally taking a progesterone for contraception. Claim 15 also defines the patient as a patient undergoing hormone replacement therapy, relieving inflammation, an in vitro fertilization program, dermatological therapy or cosmetological treatment. Claims 16 and 22, dependent upon claim 15, are also so limited. Therefore none of these claims is anticipated by either SPELLACY et al or BUTTERWORTH et al since the patient described in each of the references is not the patient covered by these method of treatment claims.

Nor does either SPELLACY et al or BUTTERWORTH et al provide a basis to reject any of these claims as obvious under 35 USC 103. There is no suggestion in either SPELLACY et al or BUTTERWORTH et al that administration according to claim 9 of a gestagen hormone together with a therapeutically effective amount

of a plasma homocysteine reducing agent to a patient undergoing treatment with a gestagen hormone composition for hormone replacement therapy, for inflammation, for an in vitro fertilization program, for dermatological therapy or for cosmetological treatment would reduce a risk to the patient of thromboembolism induced by taking the gestagen hormone. Claims 10 through 13 and 21 should be patentable over either of these two references for the same reason that claim 9 is patentable over the references. Claim 21 is especially believed to be patentable over either SPELLACY et al or BUTTERWORTH et al since claim 21 further limits the patient to a patient having an elevated plasma homocysteine level resulting from taking a gestagen hormone composition. There is not the slightest suggestion in either reference to administer to such a patient already undergoing gestagen therapy and having an elevated plasma homocysteine level resulting from the gestagen administration, a therapeutically effective amount of the plasma homocysteine reducing agent to reduce the elevated plasma homocysteine level.

The analogous situation also holds true for claims 15, 16 and 22. There is no suggestion in either SPELLACY et al or BUTTERWORTH et al that administration according to claim 15 to a patient of a gestagen hormone, undergoing hormone replacement therapy, relieving inflammation, an in vitro fertilization program, dermatological therapy or cosmetological treatment with the gestagen hormone, and whose risk of thromboembolism induced by the

gestagen hormone is reduced thanks to also taking a therapeutically effective amount of a plasma homocysteine reducing agent. Thus neither claim 15 nor dependent claims 16 and 22 would be obvious in view of either SPELLACY et al or BUTTERWORTH et al. Claim 22 is especially believed to be patentable over these references since claim 22 requires that the patient undergoing treatment with the gestagen hormone for hormone replacement therapy, relieving inflammation, an in vitro fertilization program, dermatological therapy or cosmetological treatment has an elevated plasma homocysteine level resulting from taking a gestagen hormone composition. There is absolutely no suggestion of same in either SPELLACY et al or BUTTERWORTH et al of same.

New independent claims 19 and 20 and claims 23 and 24 dependent respectively on claims 19 and 20 are also believed to be patentable over SPELLACY et al. In the methods of claims 19 and 20 the patient is a woman undergoing contraception by administration of a gestagen hormone. Furthermore the woman is also administered a therapeutically effective amount of a plasma homocysteine reducing agent that may include Vitamin B_6 which is also administered together with a gestagen contraceptive according to SPELLACY et al. Nonetheless neither of these claims is believed to be anticipated by SPELLACY et al because unlike the patients undergoing contraceptive treatment disclosed in the reference, the patients according to the method of claims 19 and 20 are healthy patients. See

Examples a, b and c. The patients undergoing contraceptive treatment in SPELLACY et al had all developed during administration of the gestagen contraceptive, adverse effects on their carbohydrate metabolism. The Vitamin B_6 was administered in conjunction with the gestagen contraceptive in order to correct the patients' imbalanced carbohydrate metabolism.

There is no indication in the SPELLACY et al reference that the Vitamin B_6 was administered to a patient, undergoing contraceptive treatment with a gestagen hormone, who was an otherwise healthy patient as in the presently claimed invention. Nor is there any suggestion in SPELLACY et al that the patient was receiving Vitamin B_6 along with the gestagen hormone to reduce the patient's level of plasma homocysteine and to thereby reduce the patient's risk of thromboembolism induced by taking the gestagen hormone. Therefore SPELLACY et al provides no basis to reject either claim 19 or claim 20 or dependent claims 23 and 24 as anticipated under 35 USC 102 or as obvious under 35 USC 103.

New independent claims 19 and 20 and claims 23 and 24 dependent respectively on claims 19 and 20 are likewise believed to be patent able over BUTTERWORTH et al. In the present invention the patient is a woman undergoing contraception by administration of a gestagen hormone. Furthermore the woman is also administered a therapeutically effective amount of a plasma homocysteine reducing agent that may include folic acid which is also administered

together with a gestagen contraceptive according to BUTTERWORTH et al. As in the case with SPELLACY et al, neither of these claims is believed to be anticipated by BUTTERWORTH et al because unlike the patients undergoing contraceptive treatment disclosed in the reference, the patients according to the method of claims 19 and 20 are healthy patients. See Examples a, b and c. The patients undergoing contraceptive treatment in BUTTERWORTH et al had all developed during administration of the gestagen contraceptive, a reversible, localized derangement in folate metabolism showing similar symptoms to cervical dysplasia, and that administration of folic acid together with the gestagen hormone improved this condition.

There is no indication in the BUTTERWORTH et al reference that the folic acid was administered to a patient, undergoing contraceptive treatment with a gestagen hormone, who was an otherwise healthy patient as in the presently claimed invention. Nor is there any suggestion in BUTTERWORTH et al that the patient was receiving folic acid along with the gestagen hormone to reduce the patient's level of plasma homocysteine and to thereby reduce the patient's risk of thromboembolism induced by taking the gestagen hormone. Therefore BUTTERWORTH et al et al provides no basis to reject either claim 19 or claim 20 or dependent claims 23 and 24 as anticipated under 35 USC 102 or as obvious under 35 USC 103.

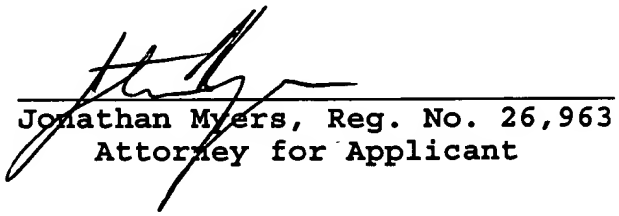
Claim 25 is believed to be neither anticipated nor obvious in view of either SPELLACY et al or BUTTERWORTH et al. In claim 25 the patient is defined more broadly than in any of the preceding claims and could include a patient as disclosed in either SPELLACY et al or BUTTERWORTH et al. However, neither of these references is believed to anticipate claim 25 because the compound administered in conjunction with the gestagen hormone to the patient is sharply defined to include Vitamin B_{12}, betaine, choline or acetyl cysteine. Administration of these compounds in conjunction with a gestagen hormone is not disclosed or suggested in either SPELLACY et al or BUTTERWORTH et al. Furthermore there is no suggestion in either reference to administer a compound that reduces the level of plasma homocysteine to a patient in conjunction with a gestagen hormone to reduce the patient's risk of thromboembolism caused by administration of the gestagen hormone. Thus no rejection of claim 25 should be made under either 35 USC 102 or 103 in view of either reference.

Now that Applicant has more sharply defined the patient undergoing treatment in amended claims 9 through 13, 15, and 16 and has similarly sharply defined the patient in new claims 19 through 24 to distinguish over the patients disclosed in SPELLACY et al and BUTTERWORTH et al, it is believed that none of these claims is inherently anticipated by the disclosure in either reference and so Ex parte Novitski and the doctrine of inherent anticipation is not

applicable. In the case of claim 25 the compounds administered to the patient in conjunction with the gestagen hormone are not the compounds disclosed in either reference and so once again the doctrine of inherent anticipation should not be applied to this claim as well.

Favorable action in this case is earnestly solicited. Applicant is enclosing authorization to charge the costs of the two extra independent claims in excess of three to the credit card of the undersigned attorneys.

Respectfully submitted,
The Firm of Karl F. Ross P.C.



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Enclosures: References
PTO 2038 (2 extra ind. claims)

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DETERMINATION OF FREE AND TOTAL HOMOCYSTEINE IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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(First received March 9th, 1987; revised manuscript received July 6th, 1987)

SUMMARY

A simple, sensitive and precise method for the determination of both free and total homocysteine in human plasma is presented. The total homocysteine in plasma consists of free homocysteine (i.e. reduced plus oxidized homocysteine in the non-protein fraction of plasma) and protein-bound homocysteine. The thiol compounds in plasma, which are reduced or liberated from plasma proteins with tri-*n*-butylphosphine, are derivatized with a thiol-specific fluorogenic reagent, ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate. The derivatives are separated by reversed-phase high-performance liquid chromatography. The concentrations (mean ± S.D.) of free and total homocysteine in plasma from 35 normal subjects were 1.94 ± 0.46 and 6.18 ± 1.19 nmol/ml, respectively.

INTRODUCTION

A sulfur amino acid, homocysteine, lies at a branch point in the metabolism scheme of methionine. Homocysteine, derived from dietary methionine, can be converted to cysteine via cystathionine in the transsulfuration pathway by two pyridoxal phosphate-dependent enzymes, cystathionine β-synthase and cystathionase. It may be remethylated to methionine [1].

Homocystinuria, an autosomal recessive inborn error of methionine metabolism, usually results from a deficiency of cystathionine β-synthase in the transsulfuration pathway. The disorder is, therefore, manifested by elevated concentrations of homocysteine and homocystine in plasma and urine. The clinical features of the disease are progressive premature arteriosclerosis and associated thromboembolic complications, mental retardation, and other tissue abnormalities [2]. The pathological accumulation of homocysteine in tissues and blood is generally considered to cause vascular complications by its injurious ef-

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fect upon the endothelial cells [3,4]. In reports about the experimental induction of arteriosclerosis in baboons by sustained elevation of the plasma homocysteine level, it was suggested that excessive homocysteine induced injuries to the endothelial cells and thereby initiated the process of premature arteriosclerosis [5–7]. This homocysteine theory of arteriosclerosis is based on clinical, experimental and epidemiological evidences. It is attractive because it might explain human arteriosclerosis attributed to both chronic pyridoxine deficiency with food processing and storage, and overloading of the transsulfuration pathway by excessive consumption of methionine-rich animal proteins [8].

Homocysteine in plasma exists as free homocysteine and as protein-bound homocysteine. So far, free homocysteine in normal human plasma has been determined by ion-exchange chromatography using an amino acid analyser [9,10] or by high-performance liquid chromatography (HPLC) with electrochemical detection [11]. However, these methods have some disadvantages with respect to sensitivity and selectivity. Furthermore, these methods neglect the existence of protein-bound homocysteine in plasma. Since Kang et al. [12] reported the presence of homocysteine bound to plasma proteins in normal individuals, total (i.e. free plus protein-bound) homocysteine in normal human plasma has been measured either by a method based on the measurement of S-carboxymethyl derivatives of plasma thiols by ion-exchange chromatography [13], or by an HPLC method in combination with a radioenzymatic assay [14]. The former method is unsatisfactory with regard to selectivity. The latter is sensitive and selective, but laborious and complicated so that it is not suitable for clinical application. Using HPLC with fluorometric detection, various reagents such as N-(9-acridinyl)maleimide (NAM), N-(1-pyrene)maleimide (PM), N-(7-dimethylamino-4-methyl-3-coumarinyl)maleimide (DACM) and monobromobimane have been tried for measuring biological thiols [15–17]. However, these methods did not have enough sensitivity or selectivity to quantify plasma homocysteine. For HPLC with fluorometric detection, very little data have been reported regarding the concentration of homocysteine in plasma.

Recently, a new fluorogenic thiol-specific reagent, ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBD-F) has been developed for the measurement of biologically important thiols [18]. Because its derivatives showed a high fluorescence and excellent stability, it was applied to the measurement of free cysteine [19] and captopril in plasma [20]. This paper describes a simple but sensitive and selective HPLC method for the determination of free and total homocysteine in plasma using a pre-column labelling agent, SBD-F. With this method, both free and total homocysteine in normal human plasma were measured.

EXPERIMENTAL

Materials

SBD-F was purchased from Wako (Kyoto, Japan) and D,L-homocysteine was obtained from Sigma (St. Louis, MO, U.S.A.). L-Cysteine and HPLC-grade

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methanol for the mobile phase were purchased from Nakarai (Kyoto, Japan). All other chemicals were of analytical-reagent grade.

Apparatus

HPLC was performed with a Shimadzu LC-6A system with two pumps and an SCL-6A system controller for solvent mixing (Shimadzu, Kyoto, Japan). Samples were introduced with a Rheodyne 7125 injection valve fitted with a 20-μl sample loop (Rheodyne, Cotati, CA, U.S.A.). Separation was carried out at ambient temperature with an analytical column, Shim-pack CLC-ODS (150×6.0 mm I.D., 5 μm particle size), protected by an ODS guard column (50×2.1 mm I.D.). The fluorescence intensities were measured with excitation at 385 nm and emission at 515 nm, using a Shimadzu RF-530 fluorescence spectrophotometer equipped with a 12-μl flow cell. The detector signal was recorded, and the peak area was quantified with a Chromatopac C-R3A integrator (Shimadzu).

Chromatographic conditions

The following solvents were used [19]: (A) 0.1 *M* acetate buffer (pH 4.0 prepared from 0.1 *M* acetic acid and 0.1 *M* sodium acetate) containing 2% methanol (v/v); (B) 0.1 *M* phosphate buffer (pH 6.0 adjusted with 0.1 *M* sodium dihydrogen phosphate and 0.1 *M* disodium hydrogen phosphate) containing 5% methanol (v/v).

The buffers were filtered through a Type HA filter (0.45 μm, Millipore, Bedford, MA, U.S.A.), mixed with methanol and degassed just prior to use. A linear gradient from solvent A to solvent B over 15 min (0–100%) at a flow-rate of 1 ml/min was applied.

Sample preparation

About 3 ml of whole blood were collected in a Vacutainer tube containing EDTA cooled on ice. The tube was immediately centrifuged at 1000 *g* for 5 min at 4°C. For the determination of total homocysteine, one part of plasma (0.5 ml) was treated with 50 μl of 10% (v/v) tri-*n*-butylphosphine (TBP) in dimethylformamide (DMF) for 30 min at 4°C in order to reduce thiols and to decouple them from plasma proteins. The solution was mixed with 0.5 ml of a chilled solution of 10% trichloroacetic acid (TCA) containing 1 m*M* Na_2EDTA under vigorous vortexing, followed by centrifugation at 1000 *g* for 5 min. A 0.2-ml aliquot of the clear supernatant was vigorously mixed with 0.4 ml of 2.5 *M* borate buffer (pH 10.5) containing 4 m*M* Na_2EDTA and 0.2 ml of SBD-F (1.0 mg/ml) in 2.5 *M* borate buffer (pH 9.5). The mixture was incubated in a shaking water-bath for 60 min at 60°C.

After terminating the reaction, the solution was cooled in crushed ice. The reaction mixture was filtered through a 0.45-μm Millipore filter. An aliquot of 10 μl was subjected to the HPLC analysis.

For the determination of free (i.e. non-protein-bound) homocysteine, another part of freshly prepared plasma was immediately added to an equal volume of a chilled solution of 10% TCA containing 1 m*M* Na_2EDTA. The solution was mixed in a vortex mixer and centrifuged to precipitate plasma proteins. A 0.2-ml aliquot

of the clear supernatant was added to a mixture of 0.4 ml borate buffer (2.5 *M*, pH 10.5) containing 4 m*M* Na_2EDTA, with 0.2 ml of SBD-F (1.0 mg/ml) in 2.5 *M* borate buffer (pH 9.5). To reduce oxidized thiols, 10 μl of 10% (v/v) TBP in DMF were added to the mixture, and then the derivatization was performed as described above. Without the addition of TBP in the reduction procedure, free reduced homocysteine could be measured.

Standards and calculation

Standards of homocysteine and cysteine were carried through the sample preparation procedure with or without TCA. Homocysteine was dissolved in 0.1 *M* borate buffer (pH 9.5) containing 2 m*M* Na_2EDTA. The homocysteine solution was stable for at least four days. A mixture of 0.2 ml homocysteine solution, 0.4 ml of 2.5 *M* borate buffer (pH 10.5) containing 4 m*M* Na_2EDTA, 0.2 ml of SBD-F (1.0 mg/ml) in 2.5 *M* borate buffer (pH 9.5) and 10 μl of 10% (v/v) TBP in DMF was treated in a manner identical to the sample preparation (i.e. standard SBD-homocysteine without TCA).

The homocysteine solution was diluted two-fold with 10% TCA containing 1 m*M* Na_2EDTA and similarly derivatized (i.e. standard SBD-homocysteine with TCA).

The concentration of plasma homocysteine was automatically calculated by the integrator from the two-point calibration line obtained by triplicate analysis of two different solutions containing SBD-homocysteine as external standard.

Statistical methods

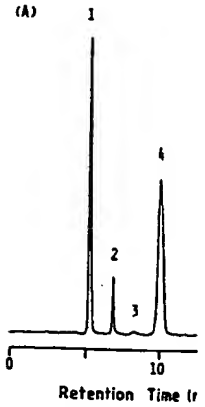
The significance of the differences between means was assessed by the Student's *t*-test. Coefficients of variation (C.V.) were calculated as standard deviations expressed as a percentage of mean values.

RESULTS

Chromatogram of plasma thiols

The present method allows the measurement of low-molecular-mass thiols in plasma simultaneously, because of the prompt derivatization with SBD-F. Fig. 1A shows a typical chromatogram for a normal person of thiols in plasma, derivatized with SBD-F. The retention times for SBD-cysteine, -homocysteine, -γ-glutamylcysteine, -cysteinylglycine and -glutathione were 5.3, 6.9, 8.5, 10, and 16 min, respectively. The retention times varied slightly with variations in room temperature and upon column aging. All the peaks could be separated to the baseline. In particular, good resolution between SBD-homocysteine and SBD-γ-glutamylcysteine was obtained using the gradient elution system as described above. SBD-homocysteine could also be separated from SBD-cysteamine, which was occasionally observed as a small peak between SBD-γ-glutamylcysteine and SBD-cysteinylglycine.

The retention time for SBD-homocysteine in the plasma sample was between 0.7 and 1 min shorter than that for standard SBD-homocysteine prepared without TCA. However, if prepared with the addition of TCA, the retention time for



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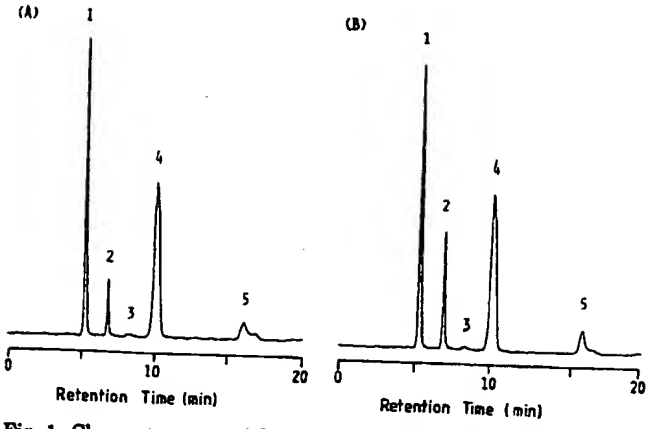

Fig. 1. Chromatograms of SBD-derivatized thiols in plasma. (A) Plasma sample from a normal person (concentrations: homocysteine 2.80 nmol/ml and cysteine 75.0 nmol/ml). (B) Mixture of the same plasma sample as (A) with 5.5 pmol of standard SBD-homocysteine (final concentrations: homocysteine 7.09 nmol/ml and cysteine 72.2 nmol/ml). Peaks: 1 = cysteine, 2 = homocysteine, 3 = γ-glutamylcysteine, 4 = cysteinylglycine, 5 = glutathione.

standard SBD-homocysteine corresponded to that for SBD-homocysteine in the plasma sample. Furthermore, the more the plasma sample was diluted with borate buffer (pH 9.5), the more the retention time for the plasma SBD-homocysteine agreed with that for the standard SBD-homocysteine prepared without TCA. Therefore, the difference in retention times between the standard SBD-homocysteine without TCA and the plasma SBD-homocysteine seemed to be attributable to the existence of numerous salts in plasma, partly due to the addition of TCA. We used the standard SBD-homocysteine with TCA for the determination of homocysteine in human plasma.

We identified these peaks as homocysteine, because the co-chromatography of plasma and different amounts of standard homocysteine, both of which were separately derivatized with SBD-F, resulted in the appearance of single, symmetrical and augmented peaks at the retention time for SBD-homocysteine in the plasma sample (Fig. 1B).

Sensitivity and linearity

Both free and total homocysteine levels in plasma were well above the detection limit of the proposed method as described below.

Over a range of the calibration curve for homocysteine (1.56–50 pmol added to the column) the response of the detector was linear. A linear regression analysis yielded $y = 245.7x - 108.8$ with a correlation coefficient of 0.99986, where y is the peak area and x is the injected amount of SBD-homocysteine (pmol). A linear relationship was also observed between the peak area of SBD-homocysteine in a plasma sample and the injected volume of the sample between 0 and 20 μl. Moreover, a linear correlation was observed between the peak area of SBD-homocysteine and the concentration of homocysteine added to human plasma.

TABLE I

RECOVERY OF HOMOCYSTEINE ADDED TO PLASMA

Different amounts of homocysteine were added to 0.5-ml aliquots of plasma ($n=5$) from a fasting normal person before adding TBP in the procedure of the assay of total homocysteine in plasma. Each value represents the mean ± S.D. ($n=5$).

Concentration of added homocysteine (nmol/ml)	Homocysteine found (nmol/ml)	Recovery (%)	Coefficient of variation (%)
0	6.82 ± 0.19	—	—
3	9.64 ± 0.32	94.3 ± 7.6	8.1
6	12.79 ± 0.33	99.6 ± 3.6	3.6
9	16.19 ± 0.50	104.2 ± 3.5	3.4
12	18.86 ± 0.31	100.4 ± 1.3	1.3

Excellent recoveries were obtained for homocysteine added to plasma (Table I).

Recovery and precision

Recovery tests were performed in several ways. When homocysteine was added to plasma either immediately after centrifugation or just before derivatization with SBD-F, the recoveries of homocysteine in the determination of total homocysteine in plasma were 99.6 and 100.5%, respectively (Table II). In the determination of free homocysteine in plasma, the recoveries of added homocysteine immediately after the centrifugation or just prior to the derivatization procedure were 100.4 and 97.2%, respectively (Table II).

The within-assay precision of the method was determined by dividing a plasma sample into nine or ten 0.5-ml aliquots and determining the plasma concentrations of free or total homocysteine in each aliquot. The C.V. values for free and total homocysteine were 3.02% ($n=9$) and 1.77% ($n=10$), respectively. The between-assay precision of the method was estimated from repeated analysis of a plasma sample, which was derivatized with SBD-F and stored at 4°C, for nine

TABLE II

RECOVERY STUDIES OF HOMOCYSTEINE

(1,3) Immediately after the centrifugation for plasma separation, homocysteine was added to plasma; (2,4) just prior to the derivatization procedure, homocysteine was added to the plasma sample treated with TCA. Each value represents the mean ± S.D. ($n=5$).

	Form of homocysteine	Concentration of homocysteine (nmol/ml)			Recovery (%)
		Initial	Calculated after addition	Found after addition	
1	Total	6.82 ± 0.19	12.82	12.79 ± 0.33	99.6 ± 3.6
2	Total	7.00 ± 0.17	11.00	11.02 ± 0.58	100.5 ± 10.6
3	Free	2.60 ± 0.08	4.60	4.61 ± 0.16	100.4 ± 8.8
4	Free	2.87 ± 0.11	4.87	4.81 ± 0.32	97.2 ± 9.9

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Concentrations of free and total homocysteine in human plasma

In twenty male and fifteen female normal fasting subjects between the ages of 19 and 39 years, we determined free, protein-bound and total homocysteine in plasma. The concentrations of free homocysteine in plasma (mean ±S.D.) were 2.06 ± 0.44 nmol/ml for men ($n=20$) and 1.79 ± 0.47 nmol/ml for women ($n=15$). The concentrations of total homocysteine in plasma (mean ±S.D.) were 6.53 ± 1.08 nmol/ml for men and 5.71 ± 1.20 nmol/ml for women. The difference of total homocysteine in plasma between sexes was statistically significant ($p<0.05$). The concentration of protein-bound homocysteine was calculated by subtracting the free homocysteine from the total homocysteine. About 70% of plasma homocysteine in both sexes was found to be protein-bound or conjugated. These data and the simultaneously determined concentrations of plasma cysteine are summarized in Table III. Positive correlations were found between the plasma concentrations of free and total homocysteine ($r=0.819, p<0.01$) and also between those of free and protein-bound homocysteine ($r=0.597, p<0.01$).

Free reduced homocysteine could also be detected in normal human plasma. Its concentration was between 0.1 and 0.35 nmol/ml, i.e. about one tenth of that of free homocysteine.

DISCUSSION

The HPLC method for the determination of plasma homocysteine described in this paper has the following characteristics and advantages.

(1) This method is a modification of the method of Toyo'oka and Imai [19] for the determination of thiols such as cysteine in plasma. This latter method involves pre-column derivatization of plasma thiols with a thiol-specific fluorogenic reagent, SBD-F, and separation on a reversed-phase HPLC column. SBD adducts have high fluorescence, giving rise to a low detection limit and excellent stability which is favourable for pre-column labelling [18]. The selectivity of SBD-F is superior to that of other labelling reagents such as bimane, because it does not react with alcohols, phenols or amino groups [16]. N-Substituted maleimides such as DACM, PM and NAM are highly fluorescent, but their adducts are unstable and give rise to two different fluorescent products [15,17]. Therefore, SBD-F is one of the most suitable reagents for the sensitive and specific determination of thiols by HPLC with fluorescence detection.

The sensitivity of our method is high, with a detection limit of 0.1–0.3 pmol for homocysteine. It is more sensitive than the methods based on HPLC with electrochemical detection [11,21–23] and than HPLC in combination with radioenzymatic assay [14]. Free reduced homocysteine, which has not been shown to be present in plasma from normal individuals by any other methods, can be detected.

(2) The proposed method utilizes a reducing agent, TBP, in order to reduce

TABLE III

DETERMINATION OF HOMOCYSTEINE AND CYSTEINE IN PLASMA

Each value represents the mean ±S.D. Homocysteine and cysteine are abbreviated to HOM and CYS, respectively.

	n	Homocysteine concentration (nmol/ml)			Cysteine concentration (nmol/ml)			Ratio		
		Free	Protein-bound	Total	Free	Protein-bound	Total	Free/total HOM	Free/total CYS	Total HOM/CYS
Men	20	2.06 ± 0.44	4.47 ± 0.78	6.53 ± 1.08*	92.6 ± 13.8	79.9 ± 18.1	172.5 ± 28.4	0.32 ± 0.05	0.54 ± 0.05	0.039 ± 0.009
Women	15	1.79 ± 0.47	3.92 ± 0.86	5.71 ± 1.20	90.2 ± 21.9	78.6 ± 13.6	168.8 ± 32.4	0.31 ± 0.05	0.53 ± 0.05	0.034 ± 0.007
Total	35	1.94 ± 0.46	4.24 ± 0.85	6.18 ± 1.19	91.6 ± 17.5	79.4 ± 16.1	170.9 ± 29.8	0.31 ± 0.05	0.54 ± 0.05	0.037 ± 0.008

*Values are significantly larger than those for women ($p < 0.05$).

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free oxidized homocysteine in the non-protein bound fraction of plasma [i.e. homocystine and cysteine–homocysteine mixed disulfide (MDS)] and to release homocysteine from plasma proteins. This enables us to measure both free and total homocysteine in plasma. Saetre and Rabenstein [11] determined the concentration of homocysteine in the non-protein fraction of plasma (i.e. free homocysteine) of five normal adults by HPLC with electrochemical detection. In this method, the disulfides were electrolytically reduced at a mercury pool electrode. However, it was pointed out that these methods were subject to the interference of reducing agents or other thiol compounds without additional preparation [24]. Recently, Refsum et al. [14] determined free and total homocysteine in plasma, using dithioerythritol as a reducing agent. However, in our thiol-labelling method, we cannot use sulfhydril-containing reducing reagents. It was reported that TBP was effective in reducing alkyl and aromatic disulfides because of its good reduction ratio [25]. We have confirmed that the release of homocysteine from plasma proteins by treating the plasma with TBP at 4°C for 30 min before deproteinization in the determination of total homocysteine in plasma was complete (data not shown).

In normal human plasma, the values of fasting free homocysteine or MDS concentrations previously reported otherwise were mostly in the range 1–3 nmol/ml [9–11,14]. Our results for free homocysteine agree with these data. The plasma concentrations of total homocysteine determined by our method, i.e. about 6 nmol/ml, are in good agreement with previously reported values using ion-exchange chromatography of S-carboxymethylated sulfhydryl compounds [13] or HPLC in combination with radioenzymatic assay [14]. So far, the radioenzymatic method of Refsum et al. [14] has been the only method by which both free and total homocysteine in plasma could be determined. The average concentration of protein-bound homocysteine, calculated by subtracting the free homocysteine from the total homocysteine, was 6.51 nmol/ml in eighteen male volunteers and 7.29 nmol/ml in sixteen female volunteers [14], which is slightly higher than our data. However, because these subjects showed a significant variation for both men and women, there is a possibility that a number of high-risk persons for arteriosclerotic disease were included, especially in the female group.

We think that it is more significant to measure protein-bound and/or total homocysteine than free homocysteine in plasma. It has been reported that the concentration of protein-bound homocysteine in plasma increases more rapidly than that of free homocysteine when rats are fed a diet deficient in vitamin B_6 [21]. Therefore, the concentration of protein-bound and/or total homocysteine in plasma rather than that of free homocysteine [26] may reflect the synthesis of homocysteine in the liver, kidneys and other tissues.

(3) With our method, it is possible to analyse not only plasma homocysteine, but also other low-molecular-mass thiol compounds in plasma, such as cysteine. The ratio between total homocysteine and total cysteine in plasma was about 0.04 in the present study (Table III). Homocysteine is converted into cysteine by two pyridoxal phosphate-dependent enzymes in the transsulfuration pathway. Therefore, excluding the possible synthesis and degradation of cysteine, the ratio between total homocysteine and total cysteine in plasma might be an indication

for vitamin B_6 deficiency, and it might reflect the activity of the transsulfuration pathway from homcysteine to cysteine. Furthermore, it might be useful for the evaluation of the treatment of homocystinuria with large doses of pyridoxine [27].

In conclusion, the method presented here is a simple, but precise, selective and sensitive determination of plasma concentration of free and total homocysteine in clinical field studies.

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INTRODUCTION

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Rapid HPLC Determination of Total Homocysteine and Other Thiols in Serum and Plasma: Sex Differences and Correlation with Cobalamin and Folate Concentrations in Healthy Subjects

Donald W. Jacobsen,[1,2,7] Vytenis J. Gatautis,[1] Ralph Green,[1,2] Killian Robinson,[3] Susan R. Savon,[2] Michelle Secic,[4] Ji Ji,[5] Joanne M. Otto,[6] and Lloyd M. Taylor, Jr.[6]

High-performance liquid chromatography with fluorescence detection has been utilized for the rapid determination of total homocysteine, cysteine, and cysteinylglycine in human serum and plasma. Our earlier procedure (Anal Biochem 1989;178:208), which used monobromobimane to specifically derivatize thiols, has been extensively modified to allow for rapid processing of samples. As a result, >80 samples a day can be assayed for total homocysteine, cysteine, and cysteinylglycine. The method is sensitive (lower limit of detection ≤4 pmol in the assay) and precise (intra- and interassay CV for homocysteine, 3.31% and 4.85%, respectively). Mean total homocysteine concentrations in plasma and serum were significantly different, both from healthy male donors (9.26 and 12.30 μmol/L, respectively; $P < 0.001$) and healthy female donors (7.85 and 10.34 μmol/L, respectively; $P < 0.001$). The differences in total homocysteine between sexes were also significant ($P = 0.002$ for both plasma and serum). Similar differences were found for cysteine and cysteinylglycine. We found a significant inverse correlation between serum cobalamin and total homocysteine in men ($P = 0.0102$) and women ($P = 0.0174$). Serum folate also inversely correlated with total homocysteine in both sexes.

Indexing Terms: *hyperhomocysteinemia/cysteine/cysteinylglycine/fluorometry/chromatography, reversed-phase/monobromobimane/sex-related differences*

Hereditary cystathionine β-synthase deficiency and certain inborn errors of cobalamin (B_{12}) and folic acid transport and metabolism can result in severe hyperhomocysteinemia and homocystinuria (*1–3*). An early recognizable clinical manifestation of homocystinuria is an unusually high incidence of premature cardiovascular disease (*4, 5*), often the cause of patient mortality (*1*). Recently, several clinical studies (reviewed in *6–10*) reported an association between milder degrees of hyperhomocysteinemia and coronary artery disease (*11, 12*), cerebrovascular disease (*13, 14*), and peripheral arterial occlusive disease (*14, 15*). In many of these studies, hyperhomocysteinemia was an independent risk factor for the cardiovascular disease (*11, 14, 15*). The etiology of mild to moderate hyperhomocysteinemia—in the absence of frank deficiency of cobalamin, folic acid, pyridoxine (B_6)—in patients with cardiovascular disease has not been established with certainty. Evidence suggests that some of these individuals are heterozygous for cystathionine β-synthase deficiency (*11, 16*). However, the phenotypic determinants currently used to establish heterozygosity show considerable overlap with normal individuals (*17*). Thermolabile methylenetetrahydrofolate reductase, an inherited enzyme defect that also results in hyperhomocysteinemia (*18*), has recently been reported to be an independent risk factor for coronary artery disease (*19*). The roles played by homocysteine in atherogenesis and thrombogenesis and the effect of therapeutic lowering of plasma homocysteine on cardiovascular disease are unknown. Accurate determination of serum and plasma concentrations of homocysteine is essential for understanding the role of homocysteine in the pathogenesis of vascular disease. Because plasma homocysteine concentrations can be lowered by administration of folic acid (*20–22*) or cobalamin (*23, 24*), assessment of homocysteine status in subjects involved in dietary modification or vitamin supplementation programs, as well as in cardiovascular disease patients at large, will require rapid and reproducible assays.

Early studies of plasma from patients with homocystinuria reported an assortment of abnormal sulfur-bearing amino acids, including homocystine and homocysteine-cysteine mixed disulfide, which, at the time, were undetectable in normal plasma (*25*). Only later were these metabolites also found in normal plasma at very low concentrations (*26–28*). It is now known that 75–90% of the homocysteine in normal plasma is covalently bound to plasma proteins by disulfide bonds (*29–31*). To determine total plasma homocysteine—the sum of all protein-bound forms, oxidized low-molecular-mass forms, and free reduced homocysteine—it is necessary to reduce disulfide bonds.

An early radioenzymatic method for total plasma homocysteine included dithioerythritol as the reducing agent to break disulfide bonds (*30*). More recent methods for determining total plasma homocysteine have utilized 2-mercaptoethanol as the reducing agent and

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gas chromatography–mass spectrometry (*32*); sodium borohydride as the reducing agent and monobromobimane as the thiol-specific fluorochromophore, followed by HPLC with fluorescence detection (FD) (*31, 33, 34*); tri-*n*-butylphosphine as the reducing agent and ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate as the fluorochromophore, followed by HPLC-FD (*35–37*); sodium borohydride as the reducing agent and HPLC with electrochemical detection (ED) (*15*); or dithiothreitol as the reducing agent, followed by automated ion-exchange chromatography (*38*) or HPLC and thiol-specific postcolumn derivatization with ultraviolet absorbance detection (UVD) (*39*).[8] Most of these methods have been reviewed in greater detail by Ueland et al. (*40*) and Jacobsen (*41*).

Here we describe a new method for the rapid determination of total plasma (or serum) homocysteine by using simultaneous sodium borohydride reduction of disulfide bonds and derivatization of sulfhydryl groups with monobromobimane followed by HPLC-FD. Based on methodology developed earlier (*31*), the new method eliminates some of the steps required in the previous method, namely, removal of excess fluorochromophore and solid-phase extraction of samples before HPLC-FD. There being no lengthy incubations and only minimal sample processing, ~85 samples plus appropriate calibrators and quality-control serum samples can be analyzed within 24 h. The method also provides quantitative information on total plasma cysteine and cysteinylglycine (CysGly). Besides describing the method, we also report its use to determine significant differences in the concentration of total homocysteine between plasma and serum and significant differences between healthy men and women in the concentrations of total homocysteine, cysteine, and CysGly.

Materials and Methods

Reagents. Monobromobimane (Thiolyte®) and L-homocysteine thiolactone were obtained from Calbiochem-Behring Diagnostics (La Jolla, CA). Monobromobimane was also obtained from Molecular Probes (Eugene, OR). Glutathione, L-cysteine, L-cysteinylglycine (CysGly), L-homocystine, sodium borohydride, 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent), Trizma base (Tris), cyanogen bromide, ethylenediamine, Sepharose 4B-CL, and *N*-acetylhomocysteine thiolactone were from Sigma Chemical Co. (St. Louis, MO). Cystine-bis-diglycine was from Serva Fine Chemicals (Westbury, NY). Perchloric acid, HPLC-grade acetonitrile, and HPLC-grade methanol were from Fisher Scientific (Fair Lawn, NJ). HPLC-grade water was produced by a Milli-Q water purification system from Millipore (Bedford, MA). Amyl alcohol was from Curtin Matheson Scientific (Houston, TX). Other chemicals were of HPLC, analytical, or reagent grade and obtained from Fisher Scientific. Sepharose-SH [Sepharose(4BCL)-ethylamido(*N*-acetyl)-homocysteine] beads were prepared as described previously (*31*) by slight modifications of the methods of March et al. (*42*) and Cuatrecasas (*43*). Briefly, this solid-phase thiol is prepared by reacting Sepharose-ethylamine with *N*-acetylhomocysteine thiolactone. The immobilized primary amine opens the thiolactone to form the amide of *N*-acetylhomocysteine and a free -SH group. Sepharose-SH is used to scavenge excess monobromobimane during the preparation of the thiol-bimane standards as described below.

Blood collection and subjects. Blood was obtained by venipuncture from fasting apparently healthy laboratory personnel. The mean (± SD) age for male donors (n = 36) was 34.4 ± 9.4 years; for female donors (n = 35), it was 33.8 ± 6.5 years (ranges 22–66 and 25–48, respectively).[9] For serum, blood was collected in evacuated tubes, allowed to clot for 1 h at room temperature, and centrifuged; the serum supernate was stored at −20°C. For plasma, blood was collected in chilled evacuated tubes containing EDTA, gently mixed, placed on ice, and centrifuged within 30 min of collection; the plasma supernate was stored at −20°C. The procedures used on human subjects in this study were in accordance with ethical standards and were approved by the Institutional Review Boards of the Cleveland Clinic Foundation and the Oregon Health Sciences University.

Determination of total serum homocysteine, cysteine, and CysGly. Serum or plasma samples (100 μL) were pipetted into 1.5-mL conical snap-cap polypropylene tubes (no. 214-3418-030; Evergreen Scientific, Los Angeles, CA) along with 10 μL of water (to compensate for volume dilution during calibration curve determination as described below) and 5 μL of *n*-amyl alcohol. The samples were then gently vortex-mixed. Sodium borohydride (35 μL of 1.43 mol/L reagent in 0.10 mol/L sodium hydroxide) was added to each tube, followed by mixing. After adding hydrochloric acid (35 μL of 1.0 mol/L) and mixing, we added 50 μL of 10.0 mmol/L monobromobimane in 4.0 mmol/L sodium EDTA (pH 7.0). (The latter reagent was prepared by diluting 0.50 mL of 50 mmol/L monobromobimane in acetonitrile with 2.0 mL of 5.0 mmol/L sodium EDTA, pH 7.0.) The tubes were capped, mixed, and incubated at 42°C for 12 min. After the samples cooled to room temperature, we added 50 μL of 1.50 mol/L perchloric acid, vortex-mixed the samples, and kept them at room temperature for 10 min. Protein was removed by centrifugation (Microfuge 12; Beckman Instruments, Fullerton, CA) at 12 200*g* for 10 min. The acidic clear supernate was adjusted to pH 4 in the centrifuge tube by adding 25 μL of 2.00 mol/L Tris, gently mixing, and centrifuging again for 1 min. An aliquot (100 μL from a final volume of 310 μL) of the supernate was transferred to a glass conical insert (no. 200-238; Sun Brokers, Wilmington, NC) contained in a 12 × 32 mm glass sample vial and then sealed with a

[8] Nonstandard abbreviations: FD, fluorescence detection; ED, electrochemical detection; and UVD, ultraviolet absorbance detection.

[9] Primary data showing donors' age; plasma and serum homocysteine, cysteine, and cysteinylglycine; and serum cobalamin, folate, and methylmalonate can be provided upon request.

10-mil (0.01-in., ~0.25-mm) Teflon disc (no. 95280; Alltech, Deerfield, IL) for HPLC analysis.

HPLC-FD. A fixed-volume autosampler (SP8875; Spectra Physics, San Jose, CA) with an 80-sample capacity was used for sample injection. Samples were kept at room temperature on the autosampler for as long as 24 h without deterioration. While on the autosampler, they were shielded from room lighting with an opaque cover. The autosampler injected 20-μL aliquots onto a 4.6 × 250 mm RP8 Ultrasphere column (no. 235332; 5-μm column packing; Beckman Instruments) equipped with Brownlee RP18 New Guard column (no. 0711-0092; 7-μm column packing; Applied Biosystems, Foster City, CA). Two Kratos Spectroflow 400 HPLC pumps (Applied Biosystems) were used to develop a methanol gradient. The buffer for pump A consisted of water: methanol:acetic acid (94.75:5.00:0.25 by vol) titrated to pH 3.40 with 5.0 mol/L NaOH. Pump B contained 100% methanol.

For routine determination of cysteine-*S*-bimane, Cys(-*S*-bimane)Gly, and homocysteine-*S*-bimane, the column was developed at a flow rate of 2.0 mL/min as follows: 0–1 min, 0% B; 1–3 min, 0–10% B; 3–9 min, 10–15% B; 9–10 min, 15–100% B; 10–11 min, 100% B; 11–12 min, 100–0% B; and 12–15 min, 0% B. If glutathione-*S*-bimane was also to be determined, an extra isocratic segment was inserted from 10 to 11 min (15% B), thus extending the overall program to 16 min. Thiol-bimane adducts were detected fluorometrically with a Kratos Spectroflow 980 HPLC detector (Applied Biosystems) with the excitation wavelength set at 390 nm and the emission wavelength >418 nm with a cutoff filter. The sensitivity range and rise time of the detector were set at 0.1 and 2 s, respectively. The fluorescence detector output was recorded on an integrating recorder (Chrom-Jet; Spectra Physics).

Calibration curves for homocysteine and other thiols. The calibration curve for total serum homocysteine was established as follows: 100-μL aliquots of quality-control sera were fortified with 10 μL of solutions containing L-homocystine of 10.0 to 1000 μmol/L. The blank consisted of sera with 10 μL of water only. The fortified and blank sera were analyzed for total serum homocysteine as described above. The integrated peak areas for homocysteine-S-bimane in the fortified samples (minus endogenous homocysteine in the blank) were plotted against the final concentration of added homocysteine. The regression analysis equation obtained from the calibration curve was used to calculate the concentration of homocysteine in normal and patients' sera. Calibration curves for cysteine and CysGly were established similarly.

Preparation of homocysteine-S-bimane and other thiol-bimane standards. L-Homocysteine-*S*-bimane was prepared from L-homocystine as follows: 0.20 mL of 0.25 mmol/L L-homocystine in 1.00 mmol/L sodium EDTA (pH 7.0) was incubated with 0.10 mL of 2.00 mmol/L monobromobimane in 1.00 mmol/L sodium EDTA (pH 7.0), 0.70 mL of 14.3 mmol/L ammonium bicarbonate containing 1.43 mmol/L sodium EDTA (pH 8.0), and 0.05 mL of 0.26 mol/L sodium borohydride in 0.05 mol/L Tris-HCl (pH 8.5) for 10 min at room temperature. Sepharose-SH beads (0.20 mL of an equivolume suspension of beads in 5.0 mmol/L sodium EDTA, pH 6.0) were added to remove excess monobromobimane. After mixing for 20 min at room temperature, we removed the beads by centrifugation, acidifed the supernate containing L-homocysteine-bimane using 0.10 mL of 1.74 mol/L acetic acid, and stored the supernate at −20°C. L-Cysteine-bimane, L-Cys(-*S*-bimane)Gly, and glutathione-*S*-bimane were prepared from L-cysteine, L-Cys(-SH)Gly, and reduced glutathione, respectively, as described above, except that 0.20 mL of 0.50 mmol/L thiol was used without sodium borohydride reduction. The thiol concentration of freshly prepared stock solutions was determined by the method of Ellman (*44*). The four acidified thiol-bimane standards were stable for as long as 3 months when stored at 4°C in the dark.

Characterization of homocysteine-S-bimane and other thiol-bimane standards. To determine the ultraviolet–visible absorbance spectra, relative fluorescence quantum yields, and fluorescence excitation and emission maxima, we purified the thiol-bimane standards by reversed-phase HPLC-UVD as follows: 0.50 mL of the standard preparations just described was injected onto a 4.6 × 250 mm RP8 Ultrasphere column with a Brownlee RP18 New Guard column, as described above for HPLC-FD. The column was developed isocratically at 2.0 mL/min by using a Beckman HPLC system consisting of two 114M pumps, a Model 421A controller, and a Model 160 absorbance detector operating at 254 nm. The solutions for pump A and pump B were also as described above. Pump volume ratios (A/B) were 91/9 for the purification of homocysteine-*S*-bimane and glutathione-*S*-bimane and 95/5 for cysteine-*S*-bimane and Cys(-*S*-bimane)Gly. Baseline-resolved peaks, detected by their absorbance and corresponding to the individual thiol-bimane standards, were collected manually. The samples were evaporated to dryness in a Savant Speed-Vac, reconstituted in 1.0 mL of buffer A (see HPLC-FD):methanol (88:12 by vol), and stored in the dark at 4°C. The thiol-bimane standards were >98% pure as judged by reanalysis with HPLC-UVD and HPLC-FD.

Comparison of methods. Random specimens from 118 individuals undergoing total plasma homocysteine determination were analyzed by the current method and by an independent method based on HPLC-ED (*15*) at the Oregon Health Sciences University.

Statistical analysis. The Wilkes–Shapiro test and examination of quintile–quintile plots were used to evaluate distribution of data. Within-subject serum vs plasma comparisons were examined by Student's paired *t*-test. Overall group comparison (e.g., male vs female plasma homocysteine) involving continuous variables that were not in a gaussian distribution were analyzed by the nonparametric Wilcoxon rank sum test. Gaussian-distributed group data were compared by using Student's unpaired *t*-test. Pearson correlation coefficients and probabilities were calculated for the relationships between serum and plasma total homocysteine and serum cobalamin and folate levels. Comparison of

results obtained by this method and an independent method for the determination of total plasma homocysteine was done by linear regression analysis as well as by the Bland–Altman procedure (*45*).

Other methods. Donors' sera were analyzed for folic acid and cobalamin with a kit assay (no. 262226; Becton Dickinson, Orangeburg, NY). In the Cleveland Clinic laboratory the reference range for serum folic acid is >2.3 μg/L (>5.2 nmol/L) and for serum cobalamin is 170 to 700 ng/L (125 to 517 pmol/L). All of the healthy subjects in this study were within reference range values for serum folate and cobalamin. Methylmalonic acid was also determined in donors' sera by stable-isotope dilution and gas-chromatography–mass spectrometry by the method of Rasmussen (*46*) except that we used 5.0 nmol of [C^2H_3]methylmalonic acid instead of 1.0 nmol. The reference range (mean ± 3 SD) for serum methylmalonic acid in healthy males and females in this laboratory is 79–376 nmol/L. Fluorescence excitation and emission spectra were obtained with an Aminco-Bowman spectrofluorometer (Model J4-8960A; SLM Instruments, Urbana, IL). Absorbance spectra were obtained with an ultraviolet–visible recording spectrophotometer equipped with a printer/plotter (Uvikon 860; Kontron, Everett, MA). We confirmed the concentration of L-homocysteine (primary standard) by amino acid analysis with a Beckman automated amino acid analyzer (Model 6300).

Results

Assay Performance

Determination of total serum homocysteine, cysteine, and CysGly. As shown in Fig. 1A, the four thiol-bimane standards are separated with near-baseline resolution; retention times are: cysteine-*S*-bimane, 6.21 min; Cys(-*S*-bimane)Gly, 6.63 min; homocysteine-*S*-bimane, 8.69 min; and glutathione-*S*-bimane, 10.73 min. A typical HPLC-FD chromatogram of normal human serum (Fig. 1B) shows retention times for cysteine-*S*-bimane, Cys(-*S*-bimane)Gly, and homocysteine-*S*-bimane of 6.20, 6.65, and 8.70 min, respectively. The homocysteine peak at 8.70 min in Fig. 1B corresponds to a serum concentration of 10.7 μmol/L. Sera from individuals with hyperhomocysteinemia, primarily from pernicious anemia, were pooled and used as high-concentration quality-control samples. The HPLC chromatogram for serum containing above-normal homocysteine is shown in Fig. 1C. The homocysteine-*S*-bimane peak (retention time 8.70 min) corresponded to a serum concentration of 52.4 μmol/L.

Calibration curves and linearity. Calibration curves were obtained by assaying normal human serum with added known amounts of L-cysteine, L-CysGly, or L-homocystine and correcting for the endogenous thiol content in the serum. The calibration curve for homocysteine (Fig. 2) was linear over the concentration range of 0–200 μmol/L for added homocysteine ($y = -3.33 + 3.38x$; $r = 0.9995$). The calibration curves for cysteine, 0–492 μmol/L, and CysGly, 0–123 μmol/L, were also linear: $y = -0.06 + 3.61x$ ($r = 0.988$) and $y = -2.75 + 3.18x$ ($r = 0.990$), respectively (data not shown). The calibration curves were used for calculating concentrations of total homocysteine, cysteine, and CysGly in unknown serum and plasma samples from healthy donors and patients.

Detection limits. The assay easily detects serum and plasma total homocysteine concentrations <1 μmol/L. In our experience, total plasma homocysteine <3 μmol/L is exceedingly rare in either patients or healthy donors. The minimal amount of homocysteine-*S*-bimane detected in the assay was 2–4 pmol (signal-to-noise ratio of 3). Because the relative quantum yields of cysteine-*S*-bimane and Cys(-*S*-bimane)Gly are similar to that of homocysteine-*S*-bimane, we estimate the detection sensitivity for these analytes to be at least 10 pmol.

Precision. The intraassay CV for total serum homocysteine, cysteine, and CysGly was determined by including normal-concentration quality-control serum (n

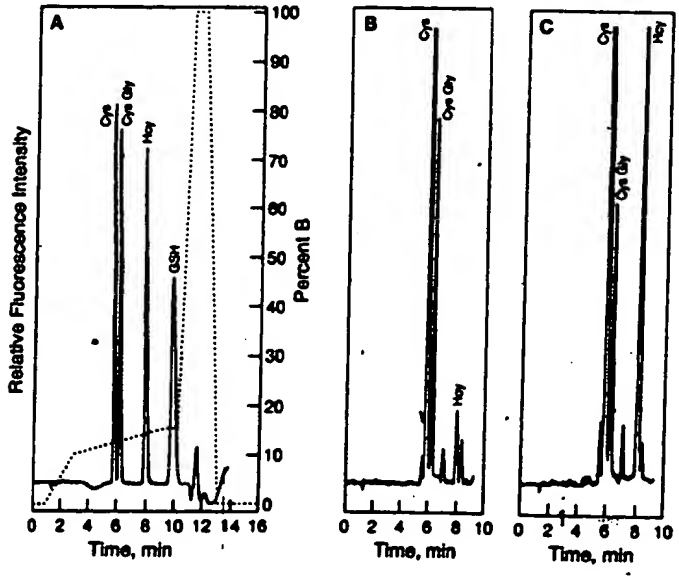

Fig. 1. HPLC-FD of thiol-bimane standards (*A*), normal human serum (*B*), and hyperhomocysteinemic serum (*C*).

(*A*) A mixture of standards, including purified cysteine-*S*-bimane (*Cys*), Cys(-*S*-bimane)Gly (*CysGly*), homocysteine-*S*-bimane (*Hcy*), and glutathione-*S*-bimane (*GSH*) was eluted from the column with a methanol gradient (—) as described in *Materials and Methods*. Total cysteine, CysGly, and homocysteine were determined in normal human serum (*B*) or in hyperhomocysteinemic serum (*C*) and analyzed by HPLC-FD as described in the text. The gradient used was identical to that in *A* except that the isocratic segment from 9 to 10 min was omitted.

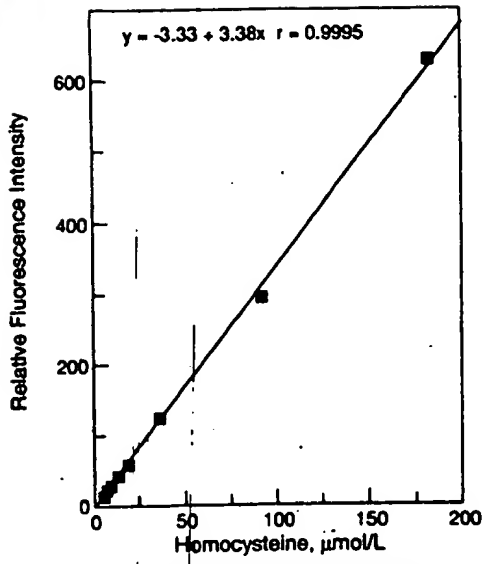

Fig. 2. Calibration curve for total serum homocysteine.

= 7) and hyperhomocysteinemic serum (n = 7) at evenly spaced intervals in a single assay of patients' samples. The interassay CV was determined by averaging the values from two normal quality-control sera and two hyperhomocysteinemic quality-control sera for seven different assays done during 1 month; the mean ± SD of the average values (n = 7) were then used to calculate the interassay CV for total serum homocysteine, cysteine, and CysGly. For total homocysteine, the intra- and interassay CVs for sera from healthy donors (means = 10.63 and 10.97 µmol/L) were 3.31% and 4.85%, respectively; for hyperhomocysteinemic sera (means = 52.8 and 56.0 µmol/L), intra- and interassay CVs were 3.78% and 3.49%, respectively. For total cysteine, the intra- and interassay CVs for normal sera (means = 208.4 and 214.5 µmol/L) were 2.35 and 3.00%, respectively; for total CysGly (means = 24.84 and 25.97 µmol/L), these were 3.96% and 7.46%, respectively.

Comparison of methods. Patients' plasma samples (n = 118) were assayed by the current method and by a previously published method based on HPLC-ED (*15*). Results from the two assays are plotted in Fig. 3A. Linear regression analysis of the data ($y = 1.19 + 0.91x$; $r = 0.974$; $S_{y.x} = 0.02$ µmol/L) showed good agreement between the two assays. Because duplicate values were available for 117 patients' samples analyzed by HPLC-FD and HPLC-ED, we analyzed the data by the Bland–Altman procedure for assessing agreement between two methods of clinical measurement. When the difference between the HPLC-ED and HPLC-FD means was plotted against the mean of the HPLC-ED and HPLC-FD means, there was good agreement and no bias between the two methods (Fig. 3B). That is, the two methods would disagree by no more than 3 µmol/L with 95% confidence.

Characterization of Thiol-Bimane Standards

The absorbance spectrum of HPLC-purified homocysteine-*S*-bimane exhibited absorbance maxima at 234 nm (relative absorbance = 1.00), 256 nm (0.603), and

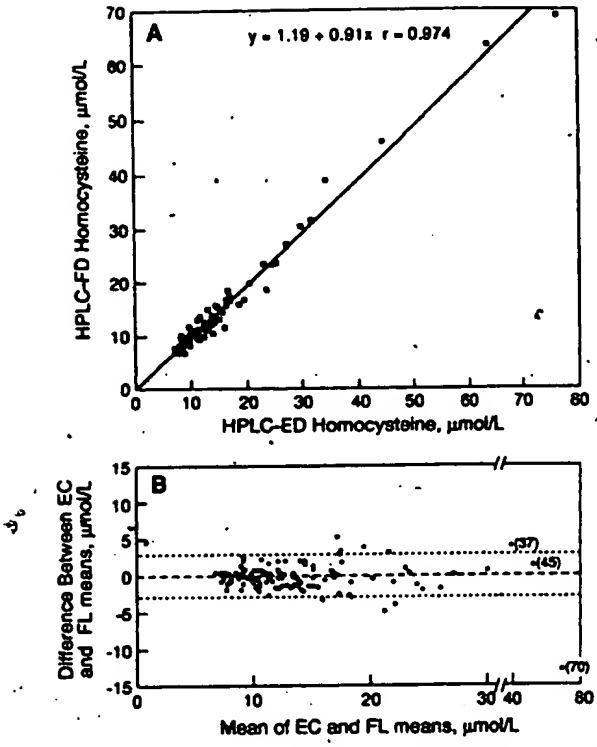

Fig. 3. (*A*) Comparison of total plasma homocysteine concentrations in patients determined by two independent methods, HPLC-FD (the current method) and HPLC-ED; (*B*) comparison of the same methods by the Bland–Altman procedure (*45*).
EC, electrochemical; FL, fluorescence.

389 nm (0.287) as shown in Fig. 4A. Similar absorbance spectra were obtained for HPLC-purified cysteine-*S*-bimane [maxima at 234 nm (1.00), 256 nm (0.593), and 389 nm (0.279)], Cys(-*S*-bimane)Gly [234 nm (1.00), 256 nm (0.582), and 389 nm (0.273)], and glutathione-*S*-bimane [234 nm (1.00), 256 nm (0.592), and 389 nm (0.279)]. The uncorrected excitation and emission spectra of homocysteine-*S*-bimane (Fig. 4B) exhibited maxima at 390 and 478 nm, respectively. Excitation and emission maxima for cysteine-*S*-bimane, Cys(-*S*-bi-

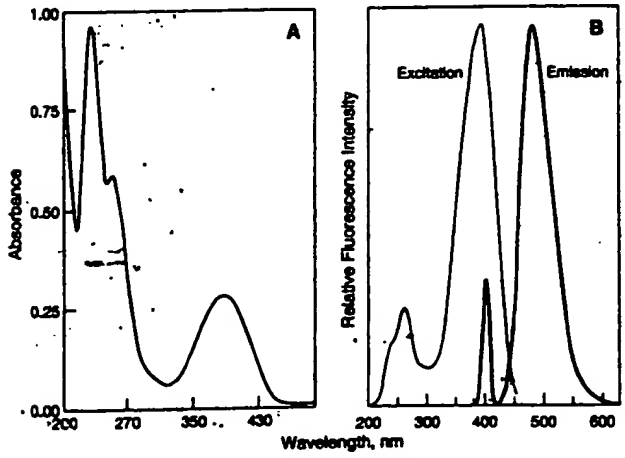

Fig. 4. Spectral characterization of homocysteine-*S*-bimane purified by reversed-phase HPLC: (*A*) ultraviolet–visible absorbance spectrum; (*B*) excitation and emission spectra.

mane)Gly, and glutathione-*S*-bimane were 392/480 nm, 392/480 nm, and 392/479 nm, respectively (data not shown). The relative fluorescence intensities of homocysteine-*S*-bimane, cysteine-*S*-bimane, Cys(-*S*-bimane)Gly, and glutathione-*S*-bimane at their emission maxima and at equivalent concentrations, based on absorbance at 389 nm, were 94.8, 97.0, 76.9, and 84.6, respectively.

Plasma and Serum Reference Values in Healthy Donors

Homocysteine. Total plasma and serum homocysteine were determined in 36 healthy men and 35 healthy women. Serum cobalamin and folate values were also determined for each donor (see footnote [9]). The plasma and serum values for total homocysteine in the men were not normally distributed, but the natural logarithm transformation of these values was. For ln-transformed values, the range (±SD) was first determined on the ln scale and then retransformed back to the original units by exponentiation. The mean (±SD) total homocysteine in plasma and serum from men (see Table 1) was 9.26 (±1.88) and 12.30 (±2.30) μmol/L, respectively, a highly significant difference (P <0.001). The ranges (mean ± 2 SD) in plasma and serum were 6.18–13.37 and 8.56–17.12 μmol/L, respectively.

In women, the plasma and serum values for total homocysteine were normally distributed, with means (±SD) of 7.85 (±2.29) and 10.34 (±3.23) μmol/L, respectively (significantly different: P <0.001). The ranges (mean ± 2 SD) for total plasma and serum homocysteine were 3.27 to 12.43 and 3.92 to 16.84 μmol/L, respectively (Table 1).

For every donor in this study, the total plasma homocysteine concentration was less than the total serum homocysteine concentration. The differences between men's and women's mean plasma and serum total homocysteine concentrations were significant: P = 0.002 for both plasma and serum (Table 1).

Men showed a significant negative correlation (Pearson) between serum cobalamin and total plasma homocysteine (r = −0.499, P = 0.0019) and between serum cobalamin and total serum homocysteine (r = −0.423, P = 0.0102). In women the negative correlation between serum cobalamin and total serum homocysteine was significant (r = −0.400, P = 0.0174), but not between total plasma homocysteine and serum cobalamin (r = −0.254, P = 0.1411). There were also significant negative correlations between serum total homocysteine and serum folate in men and women (r = −0.356, P = 0.0330; and r = −0.360, P = 0.0337, respectively) and between plasma total homocysteine and serum folate (r = −0.3059, P = 0.070; and r = −0.409, P = 0.0147, respectively). Results of linear regression analyses between serum homocysteine and serum cobalamin and folate by sex are shown in Fig. 5.

Cysteine. The plasma and serum values for total cysteine in healthy men and women were normally distributed, with means (±SD) of 209.6 (±28.42) and 266.9 (±34.23) μmol/L (P = 0.032), respectively, in men and 190.7 (±27.47) and 230.5 (±32.7) μmol/L, respectively, in women (Table 1). The plasma/serum difference was highly significant in women (P <0.001). The ranges (mean ± 2 SD) for total plasma and serum cysteine in men were 152.8 to 266.5 and 198.4 to 335.3 μmol/L, respectively, and 135.8 to 245.7 and 165.1 to 295.9 μmol/L, respectively, in women. The differences between mean plasma and serum cysteine concentrations by sex were also significant (Table 1): P = 0.006 for plasma and P <0.001 for serum.

Cysteinylglycine. The plasma and serum values for total CysGly were normally distributed in both sexes. In men the means (±SD) in plasma and serum were 30.79 (±4.48) and 40.71 (±4.99) μmol/L, respectively, significantly different (P <0.001); the mean ± 2 SD ranges were 21.83–39.74 and 30.73–50.69 μmol/L, respectively (Table 1). The means (±SD) for total plasma and serum cysteine in women were 26.53 (±3.65) and 34.10 (±4.32) μmol/L, respectively, marginally significant (P = 0.022), and the mean ± 2 SD ranges were 19.24–33.82 and 25.47–42.74 μmol/L, respectively. The sex-related differences between mean total plasma and serum CysGly were highly significant (Table 1): P <0.001 for both plasma and serum.

Table 1. Comparison of plasma and serum normal values in men and women.

			Thiol conc, μmol/L						
Variable	Group	n	Mean	SD	Min	Median	Max	Range (mean ± 2 SD)	P
Plasma homocysteine	M	36	9.26	1.88	5.94	8.71	14.98	6.18–13.37	
	F	35	7.85	2.29	4.30	7.52	14.00	3.27–12.43	0.002[a]
Serum homocysteine	M	36	12.30	2.30	8.79	11.76	20.35	8.56–17.12	
	F	35	10.34	3.23	5.89	9.97	18.89	3.92–16.84	0.002[a]
Plasma cysteine	M	36	209.60	28.42	144.00	208.30	291.40	152.80–266.50	
	F	35	190.70	27.47	117.60	191.30	258.30	135.80–245.70	0.006
Serum cysteine	M	36	266.90	34.23	163.60	267.10	353.60	198.40–335.30	
	F	35	230.50	32.70	160.90	238.40	305.10	165.10–295.90	<0.001
Plasma CysGly	M	36	30.79	4.48	22.89	30.35	43.29	21.83–39.74	
	F	35	26.53	3.65	17.24	26.08	35.19	19.24–33.82	<0.001
Serum CysGly	M	36	40.71	4.99	30.22	40.07	54.79	30.73–50.69	
	F	35	34.10	4.32	23.11	34.28	43.47	25.47–42.74	<0.001

[a] Male vs female; homocysteine values (plasma and serum) assessed by Wilcoxon's rank sum test; t-test was used for the others.

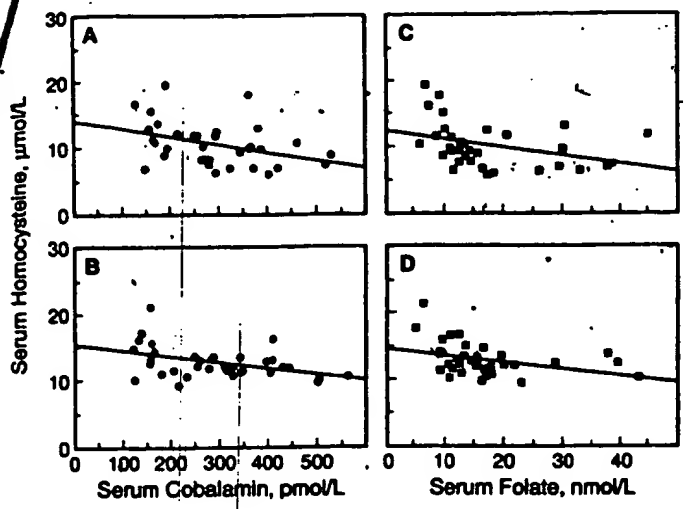

Fig. 5. Linear regression analysis of serum homocysteine vs serum cobalamin in women (*A*), serum homocysteine vs serum cobalamin in men (*B*), serum homocysteine vs serum folate in women (*C*), and serum homocysteine vs serum folate in men (*D*).

A: $y = 13.94 - 0.012x$ ($r = 0.37$; $S_{y.x} = 0.005$); *B*: $y = 15.21 - 0.009x$ ($r = 0.425$; $S_{y.x} = 0.003$); *C*: $y = 12.56 - 0.111x$ ($r = 0.344$; $S_{y.x} = 0.05$); and *D*: $y = 14.41 - 0.099x$ ($r = 0.363$; $S_{y.x} = 0.043$).

Discussion

The aims of this study were to establish a rapid and accurate assay for total homocysteine and other low-molecular-mass serum thiols in serum or plasma, to characterize the homocysteine-*S*-bimane and other thiol-bimane adducts spectrophotometrically and fluorometrically, and to establish a normal range for total homocysteine and other thiols in plasma and serum from healthy adults.

Total plasma or serum homocysteine, cysteine, and CysGly were rapidly determined by using (*a*) borohydride reduction of disulfide bonds and derivatization of thiols with monobromobimane; (*b*) perchloric acid precipitation of protein; and (*c*) HPLC-FD analysis of thiol-bimanes in the supernate. The present method, a refinement of our earlier method (*31*), has been simplified by combining the reduction/derivatization step and eliminating the reversed-phase solid-phase extraction and drying steps. Interference peaks are avoided in the new method without extensive clean-up by: (*a*) combining the disulfide reduction and thiol derivatization steps, thereby decreasing hydrolysis product formation from monobromobimane by shortening reaction and processing time; (*b*) using an excitation wavelength of 390 instead of 300 nm, thereby increasing the signal-to-noise ratio; and (*c*) using a 4.6 × 250 mm C_8 reversed-phase column instead of a 4.6 × 150 mm C_{18} reversed-phase column and a flow rate of 2.0 instead of 1.0 mL/min. Under these conditions the bimane conjugates are resolved within 8 to 9 min with little or no interference. Potentially interfering substances are retained longer than the analytes of interest but are completely eluted during column regeneration (10–13 min after sample injection; see Fig. 1A for gradient profile). All steps, including final pH adjustment of the perchloric acid supernate, are carried out in a single 1.5-mL conical snap-cap polypropylene tube. As many as 85 samples and quality-control sera can be processed in <4 h, then analyzed overnight by automated HPLC-FD. The method is sensitive, precise, and capable of detecting total plasma homocysteine over a wide concentration range.

We analyzed 118 patients' plasma samples for total homocysteine by this method and an independent method. Comparison of the two methods by linear regression analysis and the Bland–Altman procedure demonstrated very good agreement. Furthermore, the concentrations of total plasma homocysteine, cysteine, and CysGly obtained in this study are in good agreement with previously reported values obtained by others. Andersson et al. (*39*) reported total plasma homocysteine, cysteine, and CysGly in healthy men (n = 10) at 9.7 ± 2.0, 268 ± 25, and 35.8 ± 9.4 µmol/L, respectively, using postcolumn derivatization HPLC-UVD. Mansoor et al. (*47*) reported total plasma homocysteine, cysteine, and CysGly in healthy men (n = 8) at 11.85 ± 1.51, 264.3 ± 33.31, and 31.77 ± 5.36 µmol/L, respectively, using HPLC-FD.

Highly purified thiol-bimane standards were conveniently prepared from homocysteine, cysteine, CysGly, and glutathione by reacting these compounds with an excess of monobromobimane under slightly alkaline conditions, removing excess monobromobimane with Sepharose-SH, and then purifying the standards by reversed-phase HPLC-UVD. The thiol-bimane standards are useful for assessing the performance of the chromatography system and are routinely incorporated at the beginning and end of each patient run. The availability of highly purified thiol-bimane standards has made it possible to characterize the spectrophotometric properties of these compounds. The four standards had very similar absorbance spectra, with peaks at 234, 256, and 389 nm. The fluorescence excitation and emission spectra of the four compounds were also very similar, with excitation and emission maxima occurring at 390–392 and 478–480 nm, respectively. There were only minor differences in the relative quantum yields of the four thiol-bimane standards.

Total homocysteine, cysteine, and CysGly were determined in serum and plasma obtained from healthy fast-

ing donors. To standardize conditions for this study, we obtained the serum and plasma specimens from each donor at the same time, and the EDTA plasma specimen was collected in a chilled tube, placed on ice, and processed within 15 min at 4°C. The serum specimen was obtained from blood that had been allowed to clot for precisely 1 h at room temperature (22–23°C here). Significant differences were found in total homocysteine, cysteine, and CysGly concentrations between serum and plasma samples from both sexes of donors. Although the lower concentrations of the three analytes in plasma may be partially explained by fluid redistribution in plasma, this is probably not the only explanation. Erythrocytes appear to export homocysteine as a byproduct of *S*-adenosylmethionine metabolism (*48*, *49*), which may account for the higher homocysteine values found in serum from both men and women. Cellular metabolism and transport may also be responsible for the higher concentrations of total cysteine and CysGly found in serum. The significantly higher concentrations of total homocysteine in serum than in plasma and the possibility of time-dependent erythrocyte export or leukocyte release of homocysteine (and for that matter cysteine and CysGly) suggest that investigative protocols used to study the role of homocysteine in atherogenesis and thrombogenesis must be rigorously standardized.

Significantly higher concentrations of total homocysteine were found in serum or plasma from men (Table 1). In this study the female donors were all premenopausal, so these findings are therefore consistent with most other studies, as reviewed by Ueland et al. (*10*). In contrast, Andersson et al. (*50*) reported no significant difference in total plasma homocysteine between premenopausal women and age-matched men but found that postmenopausal women had lower values than age-matched men. Additional studies with larger numbers of individuals in every age decade should be carried out to resolve the issues of normal range and gender differences. In this study we found significantly higher concentrations of cysteine and CysGly in men, irrespective of whether the sample was plasma or serum.

There was a significant inverse correlation between serum cobalamin and total serum homocysteine and between serum folate and total serum homocysteine in both men and women. Although the donors participating in this study were apparently healthy individuals whose combined mean age was 34 years, several individuals had serum cobalamin concentrations <300 ng/L (221 pmol/L) and four donors had <200 ng/L (148 pmol/L). Because serum methylmalonic acid is a highly sensitive and specific indicator of functional cobalamin deficiency (*51*, *52*), we also determined serum methylmalonic acid in all of the donors enrolled in this study. All of the donors, including those with low normal concentrations of serum cobalamin, had serum methylmalonic acid values within the normal range. In contrast to homocysteine, serum methylmalonic acid concentrations did not negatively correlate with concentrations of serum cobalamin. Andersson et al. (*50*) also found negative correlations between concentrations of plasma homocysteine and of serum cobalamin and serum folic acid in apparently healthy individuals. Total homocysteine is clearly increased in states of frank cobalamin and folate deficiency, as reported by Stabler et al. (*53*), and in an unusually high percentage of elderly individuals with normal concentrations of serum cobalamin (*54*). Thus, total plasma homocysteine may be a highly sensitive indicator of both cobalamin and folic acid status, not only in patients with frank deficiencies of these vitamins, but in "normal" individuals as well. We are currently studying the effect of both cobalamin and folate supplementation in these donors to see whether total plasma homocysteine concentrations can be significantly lowered.

In conclusion, this rapid assay for total homocysteine, cysteine, and CysGly in plasma and serum is highly sensitive and precise and has the capability of high sample throughput. Using this assay, we have been able to accurately establish significant differences between plasma and serum samples and significant difference between the sexes.

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